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Quantitative measurement of nanoparticle uptake by flow cytometry illustrated by an interlaboratory comparison of the uptake of labelled polystyrene nanoparticles

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Abstract

Quantification of nanoparticle uptake into cells provides important information for both the assessment of novel nanomedicines and for nanosafety studies. Among several methods available to detect and/or quantify nanoparticle uptake, flow cytometry represents a robust high throughput method that allows measuring the internalisation of fluorescently labelled nanoparticles in thousands of individual cells in relatively short time. Several factors can influence and affect studies of nanoparticle uptake into cells, from the quality of the label and its stability, to the preparation of the nanoparticle dispersion, the way cells are exposed to the nanoparticles and several steps of sample preparation for flow cytometry measurement. Here we discuss the impact of all of these factors and methods to take them into account in order to avoid artefacts in the quantification of nanoparticle uptake and to ensure reproducibility. We then present a Standard Operating Procedure (SOP) for the quantification of nanoparticle uptake by flow cytometry, which has been developed within the European Research Infrastructure QualityNano by taking into account all of the described factors. Finally, we show the results obtained using the QualityNano SOP, demonstrating that with this SOP very good agreement in nanoparticle uptake measurements is achieved in independent laboratories by different operators using different instruments.

Keywords: nanoparticle uptake, flow cytometry, fluorescently labelled nanoparticles.

1. Introduction

Research on the biological interactions of nanomaterials has seen an enormous growth in the last decades as the potential of nanoparticles in different areas of applications such as medicine[1, 2] or different kind of products has become clear and therefore concerns on the safety of nanotechnology have to be adressed.[3, 4] One of the crucial issues for both their application in nanomedicine and for the assessment of their potential toxicity is whether nanoparticles enter cells. It is furthermore important to quantify the internalised amount as it allows distinguishing the applied dose from the internalised dose into cells or organisms. This is essential information since the internalised amount, rather than the applied dose, is often responsible for the biological action of nanoparticles.[5] In some cases it may even be the sub-cellular dose in specific cellular

compartments that determines certain biological responses, and ideally one would like to be able to determine the internalised dose with the necessary resolution.[6] By quantifying the internalised dose and following the uptake process over time, the full life-cycle of nanoparticles inside cells can be studied. Thereby it is possible to determine uptake kinetics, and monitor saturation or competing processes such as nanoparticle export, nanoparticle degradation and cell division.[7-10]

Different methods are available to determine nanoparticle uptake: in some cases these take advantage of a distinguishable nanoparticle chemical composition compared to the organic components of cells. For instance, metal and metal oxide nanoparticles can be easily quantified by classic analytical methods, such as inductively coupled plasma mass spectrometry (ICP-MS) or atomic emission spectroscopy (ICP-AES).[11-14] Typically, in standard measurement mode of ICP based techniques the *total* amount of a given *element* is obtained. Thus, for partially soluble nanoparticles, released ions, fragments of nanoparticles and the actual nanoparticles are indistinguishable. Furthermore, these methods are typically used to quantify uptake in populations of cells rather than in single cells. However, recent advancements in these techniques allow distinguishing ions from nanoparticles[15] and also to perform measurements in single cells.[16, 17] The major disadvantage of ICP based techniques is that the sample is destroyed by the measurement, thus prohibiting the possibility for further analysis. Non-destructive elemental analysis methods include Particle-Induced X-ray Emission (PIXE). PIXE can only determine the total elemental amount, and not the amount in nanoparticle form[18, 19] and can also be used for quantification at a single cell level. Currently, these latter methods are available mostly in specialised laboratories.

Other methods allow detection of nanoparticles by taking advantage of specific intrinsic properties of the nanomaterial. For example, the capacity of many nanoparticles to reflect light can be used to advantage to visualise and – to some extent – quantify their presence inside cells.[20, 21] Another example is the detection of magnetic nanoparticles using magnetic resonance.[22] Furthermore, some nanomaterials, such has carbon nanotubes, exhibit a characteristic Raman signal. Raman signals are easy to distinguish from the cell background and can be used to detect such nanomaterials inside cells.[23] Other optical properties exploited for imaging and detection of nanoparticles include surface plasmon resonance (e.g., for gold),[24, 25] and intrinsic luminescence (e.g., for ultra-small silica nanoparticles).[26] More recently second order harmonic generation and upconversion,[27] among other techniques, are also being exploited.

All these methods are useful to detect nanoparticles and thus to confirm uptake. However, they do not allow quantification of the internalised amount.

For polymeric and carbon-based nanoparticles and, in general, nanoparticles, which are more difficult to distinguish from the cell background, additional labelling is often required. Included among these particles are lipid-based nanovectors, such as liposomes,[28] or protein-based nanoparticles, such as albumin nanoparticles.[29] Stable isotope, radioactive isotope, and fluorescence labelling are among the most common forms of labelling.[30] Labelling opens up other possibilities to visualise and quantify nanoparticles in biological matrices and study their interactions with organisms and cells. Fluorescence labelling, for instance, allows monitoring nanoparticle uptake in living cells in real time.[31] It also provides a means to quantify nanoparticles inside cells, as the fluorescence of cells after nanoparticle uptake can be measured. The addition of a label to a nanoparticle may, however, also introduce some issues: surface labelling can, for instance, alter the interaction of the nanoparticle with the surrounding environment, as it *de facto* creates a different surface compared to the unlabelled material. This should be kept in mind when one aims to study the behaviour of the pristine material, rather than that of its (altered) surface labelled variant (Further studies may help to fully demonstrate eventual impact of the surface modification on the interaction with the environment, such as for instance an analysis of the protein corona composition for the pristine and surface labelled material). In this case, strategies for internal labelling (if available) may be preferred, thereby keeping the original surface unaltered, or at least less changed. Furthermore, the stability of the label in/within the nanoparticle needs to be determined and unreacted label needs to be removed very carefully to ensure that only the label bound to the nanoparticle is quantified.[32, 33] The presence of free or labile dyes can in fact lead to misinterpretation in quantifying the nanoparticle signal.[8] This particular issue will be discussed more in detail in the following, and examples on how to detect and discriminate signals from free dyes as opposed to nanoparticle-bound labels will be shown.

Each of the mentioned methods for uptake quantification has advantages and limits. For instance, in many cases measurements are performed on cell populations rather than in single cells. Frequently, signals can be detected, but absolute quantification is not possible unless some form of calibration is performed. Some of the methods are rather time-consuming and not yet available in common laboratories. Another common limitation is that in many cases it is difficult or not

possible to distinguish particles adhering to the cell membrane from those genuinely internalised. In general, when possible, the combination of multiple approaches for nanoparticle uptake is always beneficial.

Within this context, flow cytometry is a robust method that yields quantitative data of fluorescently labelled nanoparticle uptake into cells, and which is commonly available in most biological laboratories. Flow cytometry can be used to measure nanoparticle accumulation in individual cells. Tens of thousands of cells per sample can be readily measured in a short time, obtaining important information also on the variability of the response within a cell population. Although absolute nanoparticle numbers can only be determined upon careful calibration of the fluorescence signal, relative measurements can easily be performed to compare samples and determine dose-response curves or uptake kinetics.[7, 8]

Extensive work has been performed in the last few years to quantify nanoparticle uptake by this method. Here we summarise the practical principles of flow cytometry, and we illustrate how flow cytometry can be used for nanoparticle uptake studies. We also provide extensive details on how to address and overcome potential limits of this method. We then show how flow cytometry can be used for testing for possible contamination of free label. This and other several examples of factors that affect this kind of measurement, which could lead to mistakes if not recognised, are presented. Finally, we present data generated in three different laboratories using a Standard Operating Procedure (SOP) which has been developed within the European Research Infrastructure QualityNano. For this purpose, we have chosen carboxylated polystyrene as a model nanoparticle, already well characterised and easy to disperse also in cell culture medium containing serum.[7] The SOP has been developed taking into account all of the factors mentioned. The results generated using this SOP demonstrate that independent laboratories can now obtain highly reproducible data on nanoparticle uptake, as required to ensure quality in nanosafety testing. The SOP and approaches presented can be easily adapted to other nanoparticles and cells, as indeed has already been done for instance for silica nanoparticles.[35] Overall, provided care is taken in order to control and exclude a series of potential sources of artefacts and variability (discussed more below), flow cytometry is well suited to generate robust data on nanoparticle uptake by cells.

2. Results and Discussion

2.1. Practical principles of flow cytometry

Flow cytometry is a fluorescence-based method in which a suspension of cells (or other small objects, such as microorganisms, cellular fragments or particles) is passed across one or several lasers, in such a way that only one cell faces the laser at a defined time.[34] As each individual cell passes in front of the lasers, the light scattered by the cell in the forward and side directions is detected, together with the fluorescence signal emitted by the fluorescent species (here: nanoparticles) present in the illuminated volume. Multiple fluorophores can be detected simultaneously using different filters and laser combinations, with some instruments nowadays allowing the quantification of up to eighteen different parameters per cell. Furthermore, forward and side scattering (FS and SS, respectively), which are recorded simultaneously, provide information on the size and internal density or granularity of the objects illuminated by the lasers. Variations in the FS/SS double scatter plots in the presence of a biological response or due to cell damage yield additional information on the health of the cell population and of individual cells. Typically, 10-50k individual cells are measured for each sample at speeds between 300-1000 events/s, with all of the parameters mentioned determined for each individual cell. This clearly illustrates how large amounts of quantitative data can be recorded easily and in short time.

Appropriate gates can be set in order to exclude the signal generated by cell debris, which exhibits much smaller FS and SS as shown in Figure 1, or to select sub-populations of cells for separate analysis. It is important to stress that this method allows quantifying (in this case) nanoparticle uptake for individual cells, as opposed to many other methods where only a single value for the full population of cells may be measured. In this way, important information on the response of individual cells and on the variability within cell populations can also be obtained. For instance, it has been shown that within a cell population nanoparticle accumulation is different for cells in different phases of the cell cycle because of the processes of cell division.[15]

2.2. Flow cytometry to measure uptake of nanoparticles into cells

An example of a typical FS-SS double scatter plot together with the fluorescence intensity distribution of cells exposed to fluorescently labelled nanoparticles is given in Figure 1. After exposure to fluorescently labelled nanoparticles, the distribution of cell fluorescence shifts to

higher values as a consequence of the cells taking up nanoparticles. In many cases, as shown here, a relatively narrow distribution is obtained; however, also in this case it is evident that cells within the population have internalised varying amounts of nanoparticles (note the logarithmic scale in Figure 1C-D).

Applied dose-internalised dose curves (the internalised dose being the "response" in a dose-response curve) can be generated by exposing cells to different doses of nanoparticles for a given fixed time. Similarly, by measuring multiple samples at different exposure times, uptake kinetics can be obtained. For instance, this approach can address whether uptake saturates or competing processes, such as export, degradation or cell division are present.[7, 35] Changes in the shape of the fluorescence distribution, such as the appearance of multiple peaks, can also be monitored and are signs of variability in the response of the cells to the nanoparticles. Moreover, as mentioned earlier, changes in the FS-SS plots are indicative of cellular stress and cell damage. Other fluorescent probes can be added and measured at the same time using appropriate lasers and filters in order to combine the quantification of nanoparticle uptake with other parameters. For instance, the total DNA and DNA synthesis can be simultaneously monitored with appropriate markers. Thereby, cells in different phases of the cell cycle can be distinguished and the uptake of nanoparticles in each of the phases determined in parallel.[7]

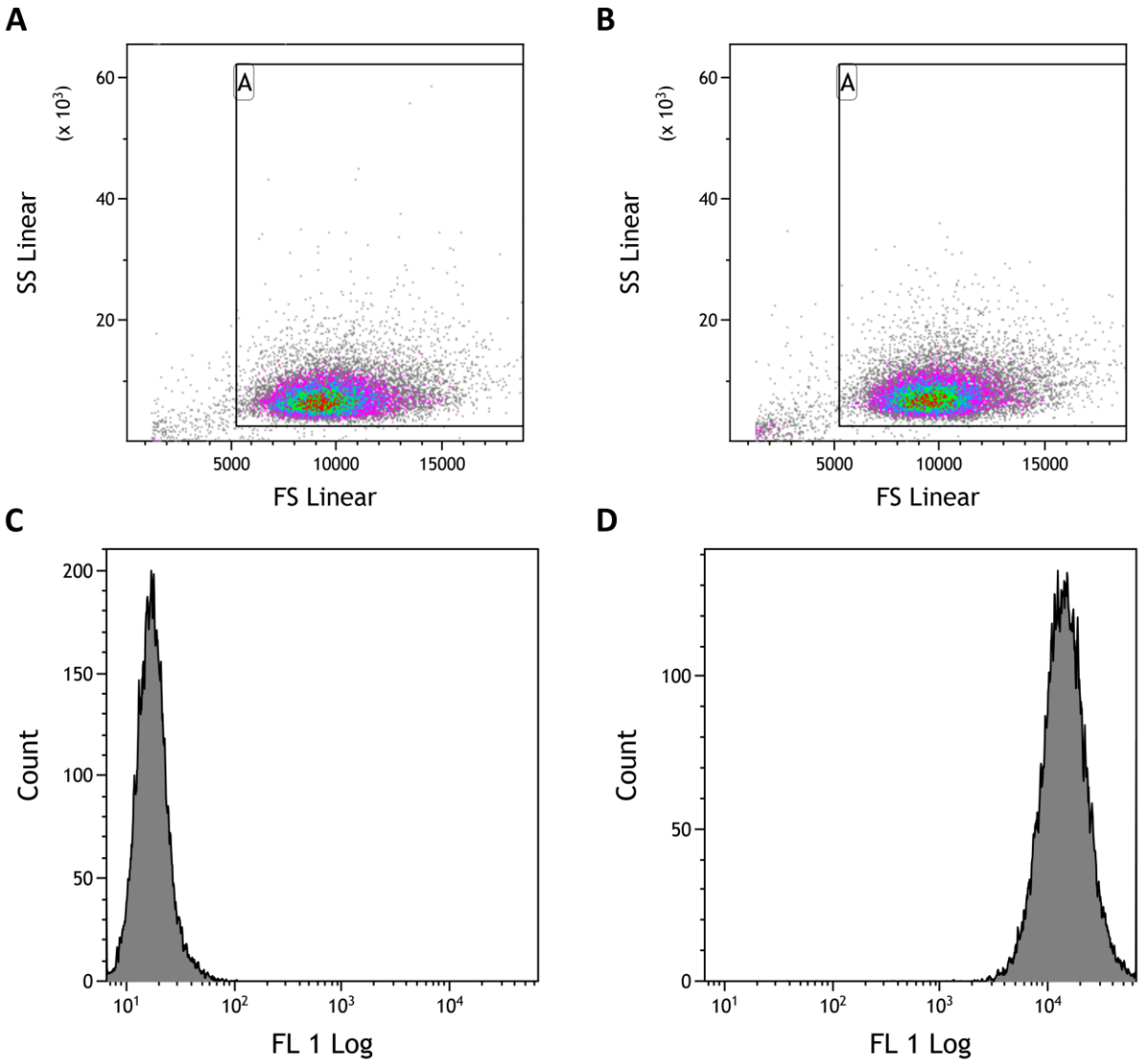


Figure 1. Typical flow cytometry results for untreated cells (left) and cells exposed to fluorescently labelled nanoparticles (right). A549 lung epithelial carcinoma cells were exposed to 100 µg/ml Yellow-Green 40 nm carboxylated polystyrene nanoparticles for 24 h. (A) and (B) Double scatter plots of side scattering (SS Linear) versus forward scattering (FS Linear) of untreated cells (A) and cells exposed to 100 µg/ml Yellow-Green 40 nm carboxylated polystyrene nanoparticles for 24 h. Healthy cells (within the A rectangle) can be easily distinguished from cell debris (outside the A rectangle) which typically has much lower FS and SS signals. In the example this is done by applying the gate A. (C) and (D) Green cell fluorescence intensity distributions (signal collected in the FL1 channel) in logarithmic scale. Untreated cells (C) have a low background fluorescence signal, while cells exposed to 100 µg/ml

Yellow-Green carboxylated polystyrene nanoparticles (D) exhibit much higher fluorescence intensity due to particle uptake.

Interestingly, some nanomaterials scatter light rather strongly and for such materials flow cytometry allows the quantification of nanoparticle uptake by detecting changes in side scattering. This effect is slightly discernible in Figure 1, although in this case it is rather small for the polystyrene nanoparticles used there. However, for other materials, such as metal and metal oxide nanoparticles, the effect can be much stronger.[36] An example of this is shown in Figure 2, which presents results for cells exposed to titanium dioxide nanoparticles. The results clearly show that the side scattering distributions shift to higher values at increasing exposure times, consistent with a higher number of titanium dioxide nanoparticles in the cells. In performing such measurements, care has to be taken to ensure that the increased cell side scattering is, indeed, due to nanoparticles, and not due to cell damage (since cell damage often leads to cell death, a cell viability test can help ruling this out). Side scattering will also be very much influenced by the chemical nature of the scattering nanoparticles and their state of agglomeration. Exercising caution, changes in side scattering can thus also be used as a measurement of nanoparticle uptake for unlabelled nanoparticles, albeit with a much lower sensitivity compared to fluorescently labelled ones.

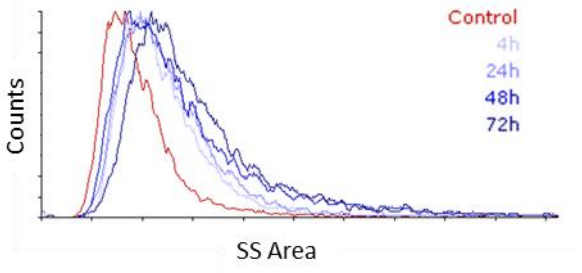


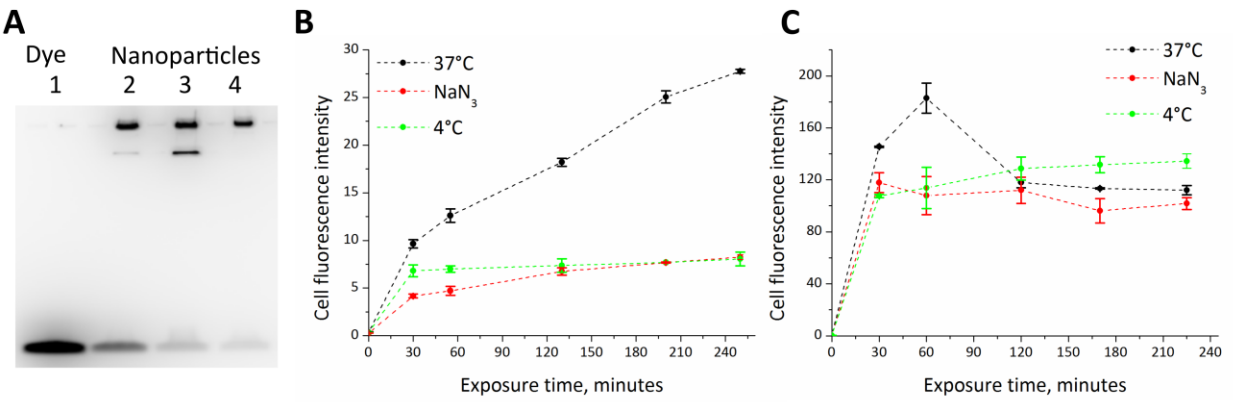
Figure 2. Measuring nanoparticle uptake by changes in cell side scattering. A549 lung epithelial carcinoma cells were exposed to 10 μg/ml carboxylated TiO_2 nanoparticles for the indicated times prior to measurement of side-scattering (SS Area) by flow cytometry**.** Control refers to untreated cells not exposed to nanoparticles.

2.3. Limits and issues of flow cytometry measurements for nanoparticle uptake

Several issues need to be taken into consideration when using flow cytometry to measure nanoparticle uptake in order to avoid creating artefacts or misinterpreting results. We will illustrate these issues for every aspect, from sample preparation to measurement.

First of all, it is essential to test both the quality of the nanoparticle labelling and the stability of the label over time. Residual free or labile dye can strongly affect the measurements of nanoparticle uptake,[8, 32] because the free dye can enter and leave the cell much more rapidly than nanoparticles, thus obscuring the signal. Even when the dye is chemically bound to the nanoparticles, residual unreacted label from the synthesis needs to be removed and can be difficult to wash off from high energy nanoparticle surfaces. Both electrostatic interactions and pi stacking can lead to strong adsorption of dyes to the surface of nanoparticles without the dye being covalently linked. Hence, standard cleaning procedures such as dialysis or centrifugation can be less effective and in need of optimisation when applied to nanoparticles.[32] Dyes adsorbed, but not covalently bound, to nanoparticles can detach once the nanoparticles are dispersed in biological fluids or inside cells, yielding false information on the location and quantity of the nanoparticle. Moreover, nanoparticle degradation in biological fluids may lead to release of dye as it has been shown – for instance - for silica nanoparticles.[33]

Size exclusion gel electrophoresis such as used by Salvati et al.[8] and shown in Figure 3A can be used to test for the presence of free or labile dye in the nanoparticles prior to their use: while nanoparticles typically are too large, free dye and fragments of nanoparticles can enter the gel. A fluorescence image reveals the presence of contaminants, separated according to their size. Furthermore, the kinetics of cellular uptake of small hydrophobic dyes differs strongly from that observed for nanoparticles: small dyes can easily enter and leave cells by passive diffusion. On the contrary, nanoparticle uptake is energy dependent and export is in most cases absent.[7, 8] Kinetic experiments by flow cytometry, such as those shown in Figure 3B-C, can thus be used to distinguish these two behaviours and reveal the potential presence of free dye.[8]



256

257 **Figure 3. Issues of free dye contamination in labelled nanoparticle samples.** (A) Fluorescence
258 images of SDS-PAGE gels can be used to detect the presence of residual free dye or degradation
259 in nanoparticle dispersions. Size exclusion allows separating the nanoparticles (on the top of the
260 gel, lanes 2-4) from smaller fragments (visible in the upper part of the gel in lanes 2 and 3) or
261 labile dye (on the bottom, lanes 1-4). Lane 1 contains free dye only. (B) and (C) Flow cytometry
262 can be used to detect the presence of residual free dye in nanoparticle samples. Kinetics and
263 energy dependence of uptake of nanoparticles (B) and free dye (C), respectively, by A549 cells.
264 Under energy depleted conditions (NaN_3) and at lower temperature, active processes of
265 nanoparticle uptake are inhibited (B). In contrast, free dyes can still enter cells by passive
266 diffusion (C). Data reproduced from Salvati *et al*.[8]

267

268 Besides issues related to the labelling of nanoparticles, cell related issues can also affect
269 nanoparticle uptake experiments. Known examples are cell cycle and cell density. As a
270 consequence of cell division, in which the internalised nanoparticle load becomes distributed
271 among the daughter cells, cells in different phases of the cell cycle show different amounts of
272 internalised nanoparticles.[7, 10] Thus, for comparability, experiments should be performed keeping
273 constant the fraction of cells in each cell cycle phase. Asynchronous cell populations typically
274 have well defined proportions of cells in the different cell cycle phases. However, cell density
275 and cell confluence are known to affect these proportions and thus should be taken into account.
276 As an example, Supplementary Figure S1 shows nanoparticle uptake results for cells seeded at
277 different starting densities. The different populations were exposed to the same nanoparticle
278 dispersions 24 h after plating. The spread of the data indicates that cell density affects

nanoparticle uptake. Another important factor is the time that cell cultures have been grown on the plate before the experiment and also the passage number since the cells were brought into culture. Typically, cell cultures are grown from frozen stocks within a certain range of passages, the optimal range of which varies depending on the cell line. It is known that the behaviour of the cells can change drastically with passage number due to differentiation events or accumulation of chromosome aberrations and spontaneous mutations that they undergo while in culture. Hence, for nanoparticle uptake studies it is important to use cells of the appropriate passage numbers. Consequently, when comparing results obtained in different experiments, passage numbers should be noted since differing numbers could be a source of variability in the outcomes.

A more general and crucial issue in experiments using nanoparticles is the preparation of the nanoparticle dispersions and their application to cells. Different nanoparticles may require different procedures to ensure that optimal dispersions are obtained. Here we want to stress that careful characterisation of the starting dispersion in cell culture media (or the media used for the study) and its stability for the duration of the experiment should be performed in order to ensure that comparable dispersions are obtained and exposed to cells each time.[37-39] If not controlled, different qualities of dispersion can lead to very different outcomes in independent experiments. Moreover, in many cases nanoparticle dispersions can age with time, including for example changes in the nanoparticle surface redox potential, surface adsorption of molecular species from the environment, bleaching of fluorescent labels etc., and the time between the preparation of the dispersion and the exposure to cells should be kept constant, as much as possible. Other factors to keep in mind – and control – are the order of mixing used to dilute nanoparticle stocks into the medium used for the experiment, since it could lead to different dispersions; the temperature of the medium used to prepare the dispersion; and the volume of dispersion added to cells. The last parameter is often not reported in literature, but needs to be specified and should be kept sufficiently high in order to exclude nanoparticle depletion in the extracellular medium due to cell uptake. Moreover, the way the dispersion is added to cells matters. Replacing the medium with the nanoparticle dispersion already prepared at the required dose seems to be preferable, while adding a small volume of nanoparticles at a high dose directly into the dish impairs proper mixing and often results in agglomeration and non-uniform exposure of the nanoparticles to the cells. This, in turn, may lead to differing uptake within the cell population, especially for short exposure times.

The medium used to prepare the dispersion also needs to be specified. First, it is imperative to use medium supplemented with serum or other biomolecules. It is known that once in contact with serum or other biofluids, a layer of biomolecules adsorb on the nanoparticle surface, forming a so-called biomolecular corona on the nanoparticles[40-42]. In the absence of a corona, nanoparticles typically interact strongly with the cell membrane,[43] in some cases killing the cell in the process.[44] Such effects are unlikely to ever occur *in vivo* because nanoparticles will always interact with cells in the presence of biomolecules. This is avoided by "passivating" the surface using supplemented biomolecules, thus creating a more realistic scenario. Second, the type of biomolecules used to supplement the cell culture medium needs to be specified, since it will change the composition of the corona on the nanoparticles. Different batches of serum, as a typical media supplement, can, in fact, lead to different levels of uptake, even for the same cells and nanoparticles, because the protein composition differs. Procedures such as heat inactivation, which also alter the serum composition, can introduce further differences if not controlled.[45] Similarly, the concentration of proteins added needs to be kept constant, since it has been demonstrated that varying the protein concentration in the medium results in very different levels of uptake.[44, 46] Finally, the type of cell culture medium also needs to be specified, because it has been shown that this also plays a role, both in the formation of a biomolecular corona and subsequent cellular effects.[47]

After exposure to nanoparticles, the next important aspects to take into consideration are those related to sample preparation for measurement. The first of these steps is the removal of the extracellular medium containing the nanoparticles prior to measurement. It is important to carefully remove all remaining extracellular nanoparticles by washing. Flow cytometry detects the fluorescence in the full illuminated volume and thus nanoparticles in dispersion or adhering externally to the cell membrane are also measured and hence affect the results. Nanoparticle adhesion to the cell membrane, or in general any surface, can be very strong.[43] Therefore, an optimisation of the washing procedure needs to be performed. Figure 4A shows an example of how to optimise the number of washes to be performed prior to measurement in order to ensure optimal particle removal.[48] In this example, after three washes the fluorescence in the washing buffer becomes much lower suggesting that (in this case) three washes are sufficient to remove the major part of the remaining particles. Performing additional washes, although they may

further remove residual extracellular nanoparticles, may risk damaging or losing cells prior to measurement.

To test how large the contribution from nanoparticles adhering externally to cells is, the adhesion of nanoparticles to the cell membrane can be explicitly measured, as shown for instance in Figure 4B. When cells are cooled to lower temperatures, active nanoparticle uptake processes are inhibited (as also shown in Figure 3).[43] This can be used to distinguish the number of internalised nanoparticles from those adhering externally to cells. In this way it was demonstrated that, although a small fraction of nanoparticles adhering to the cell membrane may remain even after careful washes and is in fact measured by flow cytometry, its contribution is almost constant, and after a few hours of uptake is much smaller compared to the internalised amount. We suggest that this small residual contamination can be neglected for longer exposure times, assuming continued nanoparticle uptake, or, better yet, explicitly measured by experiments such as those shown in Figure 4B.[43]

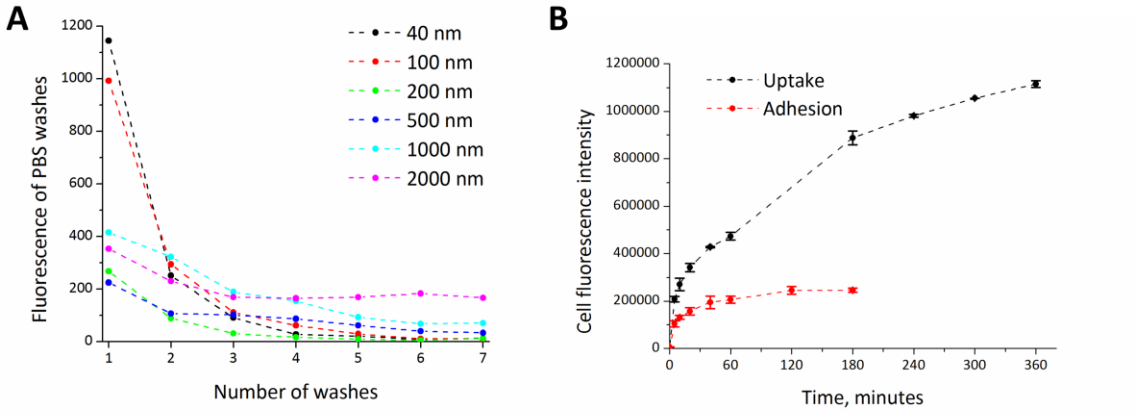


Figure 4. Optimisation of the washing procedure and measurement of nanoparticle adhesion to the outer A549 cell membrane. (A) Fluorescence intensities of the PBS used to remove extracellular Yellow-Green carboxylated polystyrene nanoparticles of the indicated sizes adhering to the dish or the outer part of the cell membrane. The results show an optimisation of the number of washes, with three washes ensuring removal of the majority of the nanoparticles. Data reproduced from dos Santos *et al.*[48] (B) Comparison of the uptake kinetics during continuous exposure at 37 °C and the adhesion kinetics at 4 °C of 40 nm Yellow-Green carboxylated polystyrene nanoparticles at 100 μg/ml in complete medium, determined by flow

cytometry. The uptake kinetics during continuous exposure was assessed by exposing cells to nanoparticles in complete medium at 37 °C for the indicated times; the adhesion kinetics was assessed by exposing cells to nanoparticles in complete medium at 4 °C for the indicated times, followed by further incubation for 3 h at 37 °C in nanoparticle-free complete medium, to ensure that nanoparticles adhering to the outside of the cell membrane had time to enter cells. This is likely somewhat of an underestimate of the adhering nanoparticles, because some nanoparticles may desorb during the 3 h of uptake. The mean cell fluorescence of 15,000 cells was determined for each replica. Data points and error bars represent the mean and standard deviation averaged over three replicas. Data reproduced from Lesniak et al.[43]

Another sensitive step in the preparation of samples for flow cytometry is cell fixation. Sometimes live cells can be measured, such as when a small number of samples are prepared and rapidly analysed, or when the cells are robust enough to be kept alive in suspension for some time prior to measurement. Care should be taken to perform the measurement as quickly as possible after sample preparation. Often one must, however, use cell fixation to overcome some of these limits, allowing preparation of a larger number of samples and longer storage times. Moreover, it allows a better control of the exposure time, since active cellular processes are halted by fixation. It is important to realise, however, that different fixatives can result in different fluorescence values being read, even for the same samples. Furthermore, even though fixation halts active processes, we have observed that especially in the first 1-2 h after cell fixation, fixed cells undergo strong changes in terms of FS and SS and also in fluorescence intensity. Because of this, it is recommended to keep the time between fixation and measurement constant for different samples within the same series. Alternatively, the magnitude of the effect should be determined explicitly by preparing multiple replicates treated all in the same way and measuring them at increasing times after fixation.

2.4. Analysis of flow cytometry results

As shown in Figure 1, flow cytometry allows obtaining cell fluorescence intensity distributions. Provided that these distributions are relatively narrow and symmetric, and that there are not multiple or very broad asymmetric peaks, the mean fluorescence intensity can be utilised as a

useful measure of nanoparticle uptake in the studied cell population. It is important to note that in such cases the cell fluorescence typically converges to the mean value already after a few thousands of cells have been measured.[7] In other words, even though flow cytometry allows measuring very large numbers of cells and obtaining well defined distributions, roughly a few thousand cells are enough to obtain the mean value for the cell population. This is particularly interesting when only low numbers of cells are available, for instance when working with primary cells.

Figure 5 illustrates this in terms of the mean cell fluorescence as a function of the number of cells measured, in which the number of cells has been artificially limited in the analysis following the measurement. It may be observed that (in this example) after 3,000-4,000 cells, the mean cell fluorescence is within 1% of the value measured for the full examined cell population (around 15,000). In other words, measurement of the remaining 11,000-12,000 cells was superfluous, as far as the mean value is concerned. Of course, in this case the number of cells was only artificially constrained and we know the "right answer" (assuming the full measured population of 15,000 cells is an accurate representation). However, for smaller populations, the same procedure may give some indication of the confidence to be had in the measured mean values. That is, by calculating how the mean changes with the number of cells, it is possible to at least get an indication of whether it appears to stabilise. For example, if only 1,500 cells of the population sampled in Figure 5 had been measured (inset), then it seems obvious that the mean has not yet stabilised, and more cells need to be measured. Other more formal procedures, e.g., jackknifing or bootstrapping,[49] can also give an indication, although the approach described here is perhaps more visually compelling.

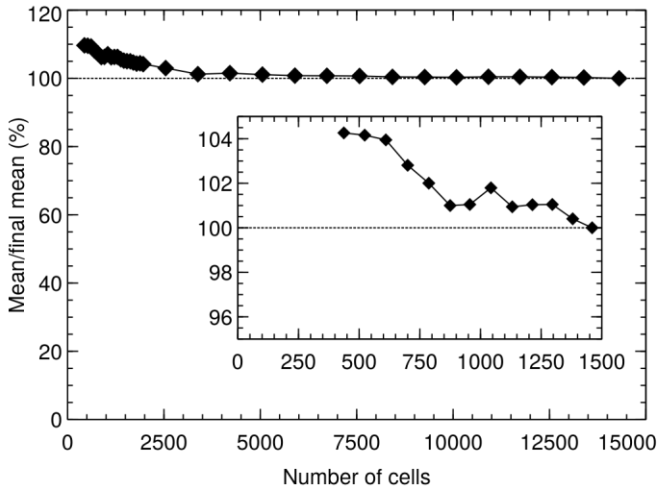
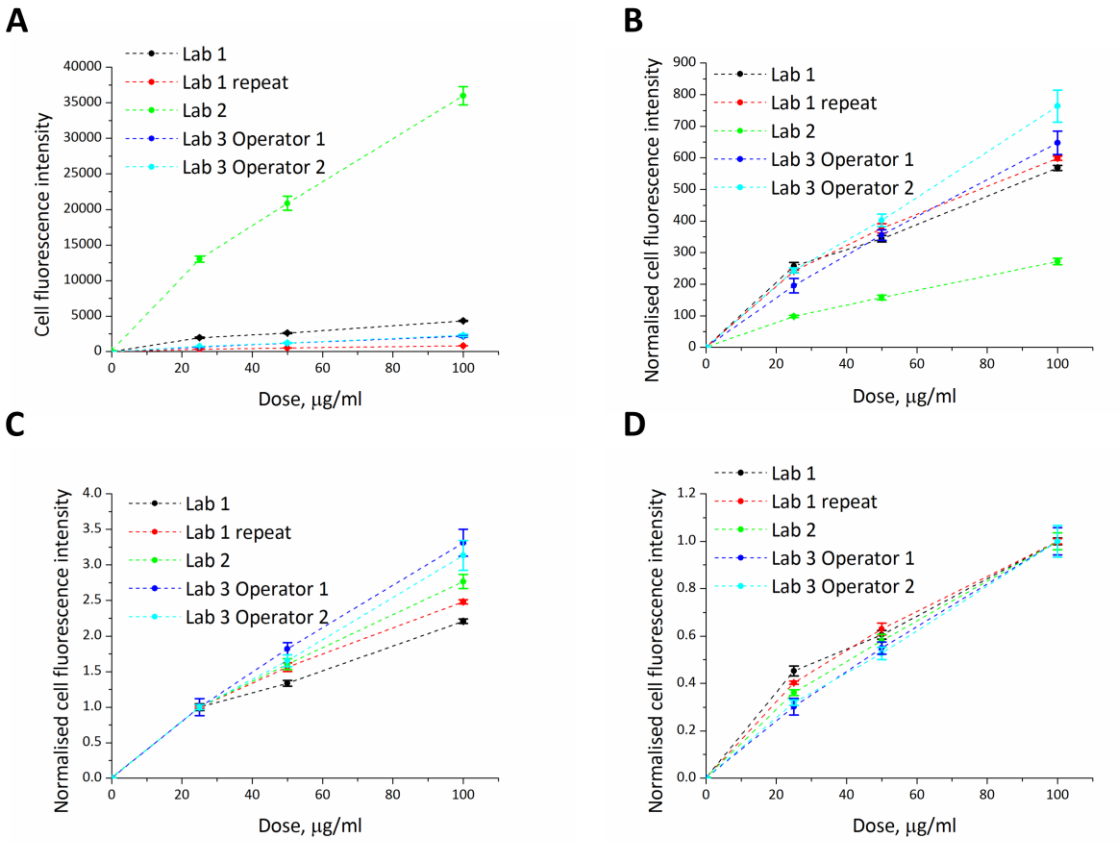


Figure 5. Mean cell fluorescence of a cell population as a function of the number of cells analysed. A549 cells were exposed to 25 µg/ml Yellow-Green 40 nm carboxylated polystyrene nanoparticles for 28 h, and around 15,000 cells from the full population of cells assessed for their fluorescence by flow cytometry. The mean cell fluorescence was calculated as a function of the number of cells "measured", by artificially restricting the number of cells taken into account in calculating the average. Furthermore, the mean was normalised by the mean of the entire examined population (~15,000 cells), to show the deviation of the (running) average to the final average. (Inset) Enlargement of the region up to 1,500 cells. In this case, the mean has been normalised by the mean calculated for around 1,500 cells, to simulate only having measured this number of cells. Data reproduced from Kim *et al.*[7]

The fluorescence measured by flow cytometry cannot be (directly) interpreted as absolute numbers of nanoparticles. Nevertheless, based on the fluorescence of a single nanoparticle comparative studies of the uptake of nanoparticles of different sizes or different fluorescence intensity may be performed,[35, 48, 50] with some accuracy.

Absolute numbers of fluorescent intensities depend on the intensity of the lasers, and the detectors and their sensitivities, which are not standardised for instruments. This makes direct comparison of results obtained across different laboratories or with different instruments challenging. A possible solution is to normalise the data. However, care should be taken when processing the data in order not to change the outcome. We illustrate this aspect on the data obtained using the Standard Operating Procedure (SOP) developed within the European Research

Infrastructure QualityNano (included in Supplementary Information), which will be discussed further below. Figure 6 shows examples of different ways of normalising the data for an experiment where the same cells were exposed to different doses of the same nanoparticles by different operators, in different laboratories and using different instruments. The data was normalised (that is, divided) by the background cell fluorescence and the fluorescence of cells exposed to the lowest and the highest dose, respectively. We observe that the unnormalised data (Figure 6A) differ greatly between data series, but nevertheless show a similar trend. Normalising the data for the background (i.e., presenting the data in terms of "fold increase") improves the situation, but only somewhat (Figure 6B). Normalisation to the first data-point where nanoparticles are present (Figure 6C) makes the data series agree at least over the lower dose range. An even better agreement is obtained by normalising to the highest dose measured (Figure 6D) which makes the different data series agree to some extent over the whole dose range. The likely reason is that if there is a relatively constant (with regards to variation in nanoparticle dose) measurement error, then the data acquired at the highest dose is, on a relative scale, most trustworthy. Thus using this value to normalise data is least prone to measurement error. Similarly, using the value at the lowest dose is better than simply the background, where the latter data is likely least precise. Further improvement may be achieved by assuming a linear relation (rather than a proportionality, as implicitly assumed when normalising with a constant factor) between the measured cell fluorescence and the number of nanoparticles. If a good model for the relation being measured exists, then fitting the data to this model is also a possible way of "normalising" the data.



458

459 **Figure 6. Data normalisation to allow comparison across different instruments or**
460 **laboratories.** A549 cells were exposed to different doses of 40 nm Yellow-Green fluorescent
461 carboxylated polystyrene nanoparticles for 24 h and their fluorescence measured by flow
462 cytometry by different operators in different laboratories using different instruments. The same
463 materials were used and the QualityNano SOP for nanoparticle uptake by flow cytometry was
464 followed. The obtained geometrical mean cell fluorescence intensities are shown here (A)
465 without any normalisation (raw data in arbitrary units) and after normalisation (that is, division)
466 by the fluorescence intensities of (B) untreated cells (background) or cells exposed to the (C)
467 lowest and the (D) highest dose of nanoparticles. Geometrical mean cell fluorescence intensity
468 values for 15,000 cells/sample were extracted from the obtained cell fluorescence histogram
469 distributions. Data are the average over 3 replicates and error bars represent the standard
470 deviation.

471

2.5. The QualityNano Standard Operating Procedure for nanoparticle uptake by flow cytometry and a Round Robin test

Based on the extensive work performed in the different participating laboratories within the European Research Infrastructure QualityNano, a detailed Standard Operating Procedure (SOP) was developed to describe in detail how to perform nanoparticle uptake measurements by flow cytometry, taking into account all of the aspects discussed above. The full SOP is included in the Supplementary Information. For this SOP, it was decided to use human A549 lung epithelial carcinoma cells, which are easy to grow and for which SOPs describing cell culture and cell growth rate determination have also been developed within QualityNano (Nelissen *et al.*, under review).

The nanoparticles used were Yellow-Green fluorescent carboxylated polystyrene nanoparticles of 40 and 100 nm from commercial sources (Molecular Probes). Extensive work has been performed with the same nanoparticles and the dispersion and stability in cell culture medium over time had already been tested.[7] Thus, they constituted an ideal model nanoparticle of high quality to allow this kind of study, excluding problems of nanoparticle agglomeration and labile dye leaching.

The SOP describes each step from cell seeding, nanoparticle dispersion and exposure to cells, up to sample preparation for flow cytometry, setting of the measurement and methods to report and analyse the results. The procedure was optimised for the chosen cells, nanoparticles and exposure times. However, the SOP can easily be adapted to other cells, nanoparticles and conditions, provided that care is taken in addressing all aspects that can affect these measurements. To this end, the tests and control experiments described in the previous sections can be used to optimise the conditions for the tested system and exclude artefacts or quality issues. For instance, for the chosen nanoparticles, vortexing of the starting stock dispersion prior to and after dilution in cell culture medium ensures preparation of a homogenous and stable dispersion. Other nanoparticles may instead require more detailed dispersion protocols. Similarly, for the chosen conditions, the developed SOP suggests three washes of the cells after exposure to the nanoparticles and uses a small number of samples. This allows using live cells for the measurements without the need to perform any fixation. However, this may need to be changed for other nanoparticles or cells.

The SOP was tested in three different laboratories. Each lab received cells, nanoparticles and serum from a common batch in order to exclude variability due to the use of materials of different sources. After defrosting cells from liquid nitrogen, experiments were performed in a restricted range of cell passage numbers, since cell behaviour and growth rate are known to be affected by passage numbers. Each laboratory performed multiple independent test runs using a dose series of the nanoparticles. In each run, the variability in nanoparticle preparation was tested by preparing three independent dispersions in cell culture medium. The percentage of cell debris was also calculated to monitor potential presence of cell damage and cell death during the preparation of the samples for measurement. For the same reason, forward/side scattering was also recorded, as were the fluorescence distributions to ensure that peak shapes were comparable and that multiple peaks were not detected.

Figure 7 shows the results obtained by the three laboratories in different independent runs. The mean cell fluorescence values have been normalised by the results for cells exposed to the highest test doses as suggested above (see Figure 6) in order to allow comparisons.

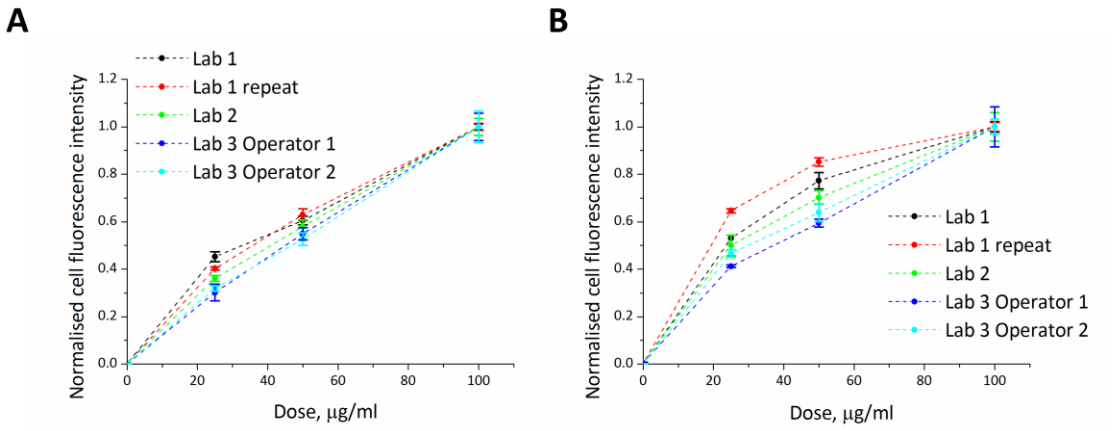


Figure 7. Comparison of nanoparticle uptake results obtained with QualityNano SOP across different laboratories. A549 cells were exposed to different doses of 40 nm (A) and 100 nm (B) Yellow-Green fluorescent carboxylated polystyrene nanoparticles for 24 h and their fluorescence measured by flow cytometry by different operators in different laboratories using different instruments. The same materials were used and the QualityNano SOP for nanoparticle uptake by flow cytometry was followed. The obtained geometrical mean cell fluorescence intensities were plotted after normalisation (division) by the fluorescence intensities of cells exposed to the highest dose of nanoparticles. For each sample 15,000 cells were measured and

mean cell fluorescence intensity values were extracted from the obtained cell fluorescence distributions. Data are the average over 3 replicates and error bars represent the standard deviation.

The results show a very good level of agreement across the different laboratories, operators and instruments used, especially for the smaller nanoparticles. A larger variability is observed for cells exposed to the larger nanoparticles. This may be due to the higher fluorescence per particle for the larger nanoparticles, which implies that a similar variability on a per particle basis will lead to a larger variability in cell fluorescence. Another possible cause includes a stronger adhesion to the cell membrane for the larger nanoparticles,[43] which implies that the detailed nature of the washing steps performed prior to measurements may matter more and lead to a larger variability when performed in different laboratories.

In general, though, these results clearly show that this SOP allows obtaining highly reproducible results in independent laboratories. It is important also to stress that while the SOP presented here was developed specifically for measuring uptake of a set of polystyrene nanoparticles, the approaches presented and experiments suggested to optimize each step of the procedure can be easily adapted for other cells and nanoparticles. Indeed similar results have also been obtained for instance for cells exposed to silica nanoparticles,[35,44] confirming that the method is well suited also to study uptake of other materials.

3. Conclusions

Flow cytometry is a technique widely available in life science laboratories, which allows measuring uptake of fluorescent nanoparticles in individual cells and generates high quality high content data with ease. Even though it does not measure absolute nanoparticle numbers in cells, it presents several advantages with respect to other methods currently available for nanoparticle uptake quantification.

Several aspects which have been reported and summarised here need to be taken into account when performing flow cytometry measurements with nanoparticles in order to exclude potential errors and artefacts related to the nature of nanoparticles and their properties. Provided these are taken into account, flow cytometry is a robust method to generate high quality data of nanoparticle uptake into cells.

A robust SOP was developed and optimised within the Research Infrastructure QualityNano based on the knowledge gained in the past years with this method. This SOP allows assessing nanoparticle uptake by cells with very good agreement between different independent laboratories and with different instruments. The same method and approaches can be easily used to implement similar SOPs for other cells, nanoparticles and conditions and measure nanoparticle uptake by flow cytometry.

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